Localization of Plasminogen Activator Inhibitor Type 2 (PAI-2) in Hair and Nail: Implications for Terminal Differentiation

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Plasminogen activator inhibitor type 2 (PAI-2) is an unusual serine proteinase inhibitor in that it is largely retained within the cell and is found in high concentrations in the upper viable layers of human epidermis. Studies using transfected cell lines that express high levels of PAI-2 have suggested that this inhibitor may confer protection against programmed cell death. To test the hypothesis that PAI-2 may protect epithelial cells *in vivo* from premature programmed cell death, we determined expression patterns of PAI-2 in murine hair and nail. These epidermal derivatives are comprised of numerous epithelial cell types with distinct differentiation pathways. Furthermore, the cyclic nature of the follicular epithelium makes it ideal for studying sequential stages of cell differentiation and death. PAI-2 mRNA and protein were detected in the differentiating cells of the outer root sheath and medulla of the follicle during the anagen phase of the hair growth cycle. PAI-2 was also detected in the permanent portion of the catagen follicle. In the telogen phase of the hair growth cycle, PAI-2 was limited to the postmitotic cells of the outer root sheath directly abutting the club hair. In the nail, PAI-2 was detected in the differentiating cells of the matrix and nail bed. This consistent, selective distribution of PAI-2 in the postmitotic, maturing cells prior to terminal keratinization and death suggests that (i) PAI-2 may be considered as a differentiation marker for many epithelial cell types, and (ii) PAI-2 is appropriately positioned to protect epithelial cells from premature demise.
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Plasminogen activator inhibitor 2 (PAI-2) is a serine proteinase inhibitor originally purified from human placenta (Bachmann, 1995; Kruithof *et al*, 1995). At least under normal circumstances, the *in vivo* distribution of PAI-2 appears rather limited, with the highest levels noted in skin keratinocytes (Kawata *et al*, 1996). PAI-2 is also found in large amounts in monocytes/macrophages, but in contrast to many serine proteinase inhibitors, it is not detectable in plasma, except during the latter stages of pregnancy (Kruithof *et al*, 1995). Although kinetic data indicate that PAI-2 is an effective inhibitor of both urokinase type plasminogen activators (uPA) and tissue type plasminogen activators, its physiologic role remains undefined (Andreasen *et al*, 1990). Plasminogen activators catalyze the conversion of plasminogen to its active form, plasmin, and thereby are responsible for the cleavage of fibrin and other extracellular matrix molecules relevant to vascular patency and tissue remodeling (Collen and Lijne, 1991; Conese and Blasi, 1995; Andreasen *et al*, 1997). As these proteolytic activities are extracellular, any candidate inhibitor for uPA and/or tissue type plasminogen activator would likewise be expected to have an extracellular distribution. Surprisingly, PAI-2 is in many cases not secreted but is rather concentrated intracellularly (Medcalf *et al*, 1988; Belin *et al*, 1989), leading to speculation that it has roles other than inhibition of plasminogen activators.

Hints to a possible intracellular function for PAI-2 have come from examination of cell lines transfected with PAI-2 cDNA (Kumar and Baglioni, 1991; Dickinson *et al*, 1995). Such cell lines, which produce large amounts of active PAI-2 and retain most of it in the cytoplasm, are resistant to programmed cell death induced by several but not all stimuli. These data have led to the hypothesis that PAI-2 may function intracellularly to protect cells from programmed cell death.

PAI-2 mRNA and protein are present in the more differentiated epithelial cells of human epidermis and hair follicle (Hibino *et al*, 1988; Lyons-Giordano *et al*, 1994). Furthermore, studies with human keratinocytes in culture have shown that PAI-2 has characteristics of a differentiation antigen, at least some of which is incorporated into the squame-like cells (Jensen *et al*, 1995; Robinson *et al*, 1997). Based on these data and the transfected cell line experiments described above, we have hypothesized that PAI-2 may protect the differentiating keratinocyte from undergoing the final stages of terminal differentiation and death. A premature demise, prior to the time when the keratinocyte has elaborated all of the differentiation products necessary to generate a fully developed stratum corneum or hair shaft, would obviously have very deleterious effects on the organism.

The hair follicle is an ideal model with which to test further our hypothesis that PAI-2 may play a role in terminal differentiation, because the cyclic nature of this epidermal derivative provides numerous opportunities to study events involved in epithelial maturation and terminal differentiation (Lavker *et al*, 1998). Hair follicles undergo cycles of growth (anagen), involution (catagen), and rest (telogen),

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Abbreviations: PAI-2, plasminogen activator inhibitor type 2; uPA, urokinase type plasminogen activator.

which in juvenile mice can be easily studied because they are highly synchronized and well timed (Wilson *et al*, 1994). During anagen, the matrix keratinocytes in the bulb region proliferate rapidly (Van Scott *et al*, 1963; Weinstein and Mooney, 1980); however, upon becoming postmitotic they differentiate into the inner root sheath, as well as the cuticle, cortex, and medulla of the hair shaft (Reynolds and Jahoda, 1993). At the end of anagen, matrix keratinocytes abruptly cease proliferating and enter a phase of rapid programmed cell death, so that the lower follicle involutes and regresses (Chase, 1954; Dry, 1962; Seiberg *et al*, 1995). This stage is known as catagen. At the end of catagen, the follicle enters a period of rest (telogen), characterized by extremely low proliferative rates and no further growth of the hair shaft (Wilson *et al*, 1994). The telogen hair has a club-shaped proximal end within the hair follicle, which in mice and rats is not lost until a new hair is fully formed from a subsequent anagen (Chase, 1954; Dry, 1962). Retention of the telogen hair is essential because a dense, intact coat of hair functions in a thermoregulatory capacity and as a first line of defence from abrasions, properties vital for the survival of these animals.

In this study we have determined the protein and mRNA expression patterns of PAI-2 in the various epithelial cells that constitute the murine follicular epithelia during the three phases of the hair growth cycle. We have also studied the distribution of PAI-2 in the murine nail apparatus, an epidermal derivative that bears many similarities to the hair follicle (Dawber and Baran, 1984; Baden and Kvedar, 1993). We report that PAI-2 is consistently expressed in the more differentiated keratinocyte populations of each of these tissues, consistent with a role in protecting cells from premature cell death.

MATERIALS AND METHODS

Anti-PAI-2 immunohistochemistry Anti-mouse PAI-2 anti-serum was prepared by injection of a rabbit with mouse PAI-2:GST fusion protein that had been purified from inclusion bodies by 8 M urea extraction and glutathione-Sepharose chromatography. The anti-serum was absorbed with sham transfected bacterial lysates to remove any cross-reactivity with either bacterial or GST antigens. The IgG fraction was isolated using a Protein A column. Western blots showed that this anti-serum recognized recombinant mouse PAI-2 as well as a single band in mouse epidermal extracts at 43 kDa, the size of mouse PAI-2 (Wohlwend *et al*, 1987).

For staining, skin from the nape of the neck and hind feet was removed from mice of the indicated ages, fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.0, washed and incubated overnight in 80% ethanol, and then processed for paraffin embedding and sectioning (6 μm). After dehydration, sections were fixed again in 4% paraformaldehyde in PBS pH 7.0; blocked for 1 h at room temperature with 10% goat serum in PBS; and incubated overnight at 4°C with rabbit anti-mouse PAI-2 IgG or normal rabbit IgG as a control (5 μg per ml in PBS containing 10% goat serum). After washing in PBS, slides were incubated with biotinylated goat anti-rabbit IgG (Vectastain Laboratories, Burlingame, CA) for 1 h at room temperature, washed, incubated with avidin-biotin-peroxidase reagent (Vectastain) for 45 min at room temperature, and washed again. Staining was then visualized by reaction with diaminobenzidine for 10–15 min at room temperature, according to manufacturer's directions (Sigma, St Louis, MO). Slides were washed a final time in PBS, stained with hematoxylin (Gill #1), dehydrated, mounted with permount and coverslipped. Mice of several strains (C57Bl/6, C3H/129 hybrids, Sencar) were utilized and no differences were noted among strains.

PAI-2 *in situ* hybridization A 930 bp Pst1/PvuII fragment of mouse PAI-2 (Belin *et al*, 1989) was subcloned into pBluescript SK± at EcoR1/HindIII; digestion with HindIII and transcription with T3 were utilized for anti-sense probes, and digestion with SmaI and transcription with T7 were utilized for sense probes. RNA probes were prepared using 35S-labeled UTP. *In situ* hybridization was conducted as described (Keeton *et al*, 1993; Jensen and Lavker, 1996) using paraffin sections of skin and nail.

Transmission electron microscopy Skin was fixed and processed for transmission electron microscopy as described previously (Cotsarelis *et al*, 1990).

RESULTS

PAI-2 is expressed in the hair follicle The hair follicle comprises at least seven types of morphologically and/or biochemically distinguishable keratinocytes that spatially organize and coordinately differentiate to form the outer root sheath, inner root sheath, and hair shaft (Hashimoto and Shibazaki, 1976; Reynolds and Jahoda, 1993; Kamimura *et al*, 1997). Immunohistochemical and *in situ* hybridization experiments revealed that PAI-2 was present only in specific subpopulations of cells, dependent upon the phase of the hair growth cycle. In the adult mouse, the vast majority of hair follicles at any given time are in the telogen (i.e., resting) phase, where the hair shaft is no longer growing, but must be retained in the follicle. During telogen, PAI-2 antigen and mRNA were found exclusively in a single layer of nucleated, outer root sheath keratinocytes that were adjacent to and organized around the hair shaft (**Fig 1**). In cross-section, the PAI-2-stained cells appeared as a prominent ring encircling the hair shaft (**Fig 1**, *inset*). These cells were consistently at least one cell layer removed from the basal cell population, which is contiguous with the basal layer of epidermis and constitutes the proliferative layer. Within these outer root sheath cells the PAI-2 staining pattern was predominantly cytoplasmic. The PAI-2-stained keratinocytes displayed ultrastructural features that were typical of viable cells (**Fig 2*a*, *b***). They possessed a large nucleus with prominent chromatin; no evidence of nuclear fragmentation, clumped chromatin, or nuclear membrane blebbing was apparent. The cytoplasm was filled with ribosomes and copious bundles of keratin filaments. Lysosomes, autophagic vacuoles, and lipid droplets, features associated with dying cells, were not present. These PAI-2-staining cells interlocked with the remnants of the inner root sheath, along a highly interdigitating interface, suggesting that they were involved in anchoring the hair shaft to the base of the follicular epithelium. Even after the follicle re-entered the growth phase, leading to initiation of a new hair shaft, the cells surrounding the old hair shaft (club hair) retained PAI-2 (**Fig 3*a***).

In the newly forming anagen hair follicle, whether during the first or subsequent hair growth cycles, PAI-2 was initially very difficult to detect; however, later in anagen, PAI-2 staining was detectable in a single line of cells beginning near or above the critical line of Auber (marking the widest diameter of the follicle; Auber, 1950–1951) and extending upward toward the mid-region of the follicle (**Fig 3*b***). The region below this line is of significance because it is the site of the majority of proliferative activity in the follicle, and little or no PAI-2 mRNA or protein was observed in the matrix keratinocytes of this region. In the mid-region of the follicle (**Fig 3*c***), it became apparent that PAI-2 was present exclusively in a single layer of very flat cells, which abuts the inner root sheath. This layer has previously been described as the "companion" layer (Orwin, 1971) or the innermost layer of the outer root sheath (Ito *et al*, 1986; Ito, 1988). Closer to the skin surface, near the infundibulum (**Fig 3*d***), where the inner root sheath is totally disintegrating, these innermost outer root sheath cells assume an irregular shape with a serrated surface facing the hair shaft. Interestingly, these cells initially retained their nuclei, consistent with earlier findings (Ito, 1988) that, in the mouse, these cells do not keratinize until a very late stage when the inner root sheath has completely degenerated. These irregularly shaped cells also initially retained strong staining for PAI-2 (**Fig 3*d***), but then lost detectable PAI-2 when they moved slightly farther up the follicle and finally began to keratinize. Hence the innermost cells of the outer root sheath contained PAI-2 along almost the entire length of the follicle, until they underwent terminal differentiation and death near the infundibulum.

PAI-2 was also expressed during anagen by a second subset of cells, the medulla cells, which form a vertical column in the center of the hair shaft (**Fig 3*c–h***). The medulla cells in the keratogenous zone, just above the bulb, are the least differentiated, and upward vertical movement is coordinate with increasing differentiation. Only a subpopulation of medulla cells, i.e., those in the mid-region of the follicle, contained PAI-2 mRNA (**Fig 3*f*, *g***); intense staining for PAI-2 antigen was also detected in this region (**Fig 3*h***). Newly forming medulla cells in and just above the keratogenous zone contained neither PAI-2 antigen nor mRNA (**Fig 3*f*, *g***). Furthermore, the highly differentiated medulla cells approaching the skin surface expressed no PAI-2 mRNA (**Fig 3*f***), although weak immunostaining for PAI-2 was often observed (**Fig 3*h***), suggesting retention of this protein. The extremely intense and highly localized signal for PAI-2 mRNA, which is mirrored by the antigen, indicates an acute induction of synthesis of the inhibitor during a very specific stage in medulla cell differentiation. In contrast,

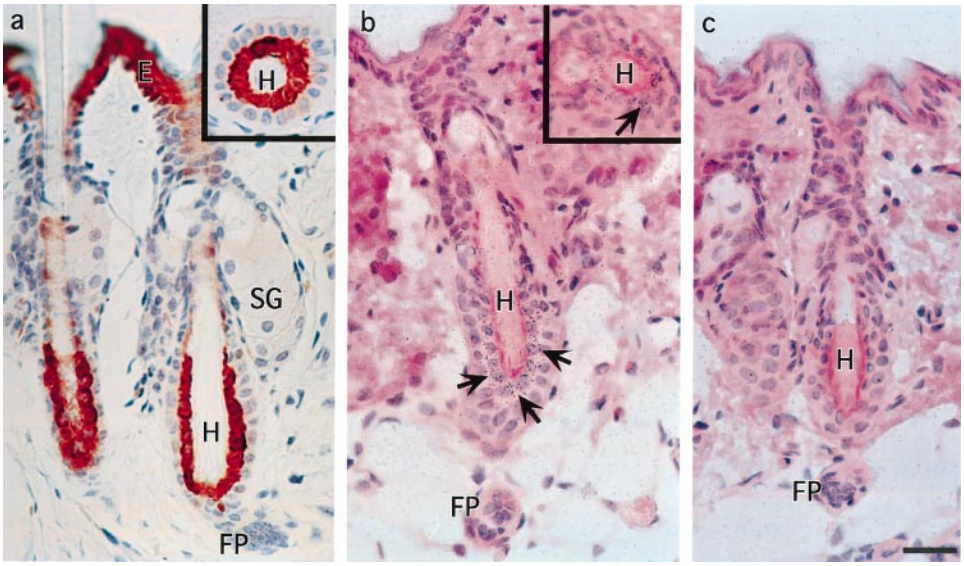

Figure 1. PAI-2 is expressed in the outer root sheath of telogen follicles. Skin from mice in the telogen phase of the hair growth cycle was processed for immunostaining with rabbit antibody against mouse PAI-2, using avidin-biotin-peroxidase and diaminobenzidine detection (*a*), or for *in situ* hybridization with ^{35}S-labeled anti-sense (*b*) or sense (*c*) probes to PAI-2. Longitudinal sections of telogen follicles are shown in (*a*)–(*c*); the insets show cross-sections. PAI-2 mRNA and antigen are detected in a single layer of cells that surrounds the hair shaft (H). SG, sebaceous gland; FP, follicular papilla; E, epidermis. *Scale bar*, 20 μm.

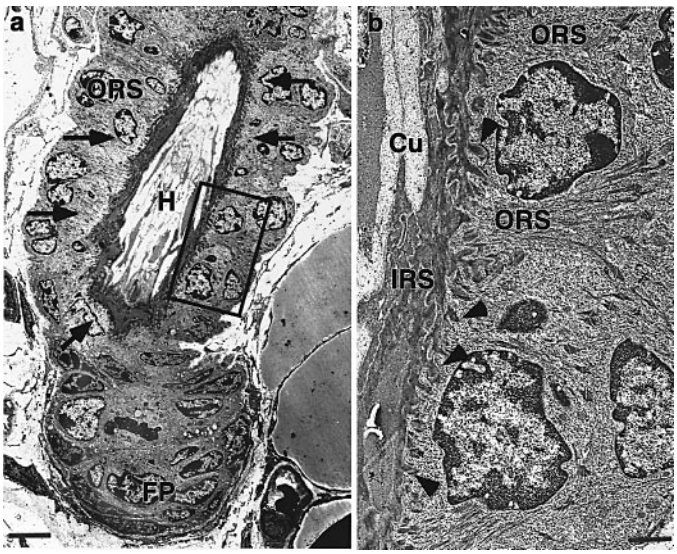

Figure 2. Outer root sheath cells during the telogen phase of the hair growth cycle show no evidence of degeneration. (*a*) Ultrastructure of the lowermost portion of a follicle during the telogen phase of the hair growth cycle showing the relationship between the outer root sheath cells (ORS) and the hair shaft (H). *Arrows* point to those cells immediately adjacent to the hair shaft that, as shown in **Fig 1**, express PAI-2. The area within the rectangle is shown at a higher magnification in (*b*). FP, follicular papilla. *Scale bar*, 5 μm. (*b*) Outer root sheath cells (ORS) immediately adjacent to the fully cornified inner root sheath (IRS) appear viable as evidenced by their intact nuclei and well organized cytoplasm filled with abundant ribosomes. The highly interdigitating boundary (*arrowheads*) between the ORS cells and the IRS suggests that these two follicular regions form a tight complex. The hair shaft cuticle (Cu) also interlocks with the IRS anchoring the telogen hair. *Scale bar*, 1 μm.

the PAI-2 mRNA in the outer root sheath during telogen appears to be constitutively present (**Fig 1*b***), albeit at a lower level.

During catagen (**Fig 3*i*, *j***), there is very rapid degeneration of the bulb and lower end of the follicle, which shrinks to approximately one-third its original size (Chase, 1954; Dry, 1962). PAI-2 was strongly expressed in catagen follicles, in a population of epithelial cells just beneath the hair shaft that represent the permanent portion of the follicle and most likely secure the hair shaft. Little or no PAI-2 was detected in the thin, wispy, strands of cells within the connective tissue sheath that comprised the lower part of the catagen follicle and were destined to die.

PAI-2 is expressed in the nail structure Like the hair follicle, the nail apparatus is comprised of several epithelial cell types, each of which follows a unique differentiation pathway (Dawber and Baran, 1984; Baden and Kvedar, 1993) and several of which stained for PAI-2 (**Fig 4**). In the matrix cells, whose terminal differentiation product is the nail plate, PAI-2 was detected in all layers except for the proliferative basal layer (**Fig 4**). Most intriguingly, the staining for PAI-2 gradually became fainter and was then lost altogether just as the cells lost their nuclei and underwent the final stages of terminal differentiation to generate the fully keratinized nail. PAI-2 was also strongly detected in the epithelial cells that formed the nail bed (i.e., that epithelium that lies beneath the nail plate and anchors it). Similar to the matrix epithelium, only the differentiated cells, and not the basal cells, of the nail bed stained for PAI-2.

DISCUSSION

Localization of PAI-2 to specific subsets of cells within the diverse epithelial cell types that comprise the hair follicle and the nail apparatus has functional implications relating to the hypothesis that this inhibitor endows cells with resistance to terminal differentiation, a highly specialized form of programmed cell death (McCall and Cohen, 1991; Polakowska and Haake, 1994). In no case is PAI-2 observed in the proliferative precursor cells that give rise to any components or structures in the hair follicle or nail apparatus. Specifically, PAI-2 is not expressed in the highly proliferative matrix keratinocytes of the follicular bulb that give rise to the inner root sheath and the hair fiber, nor in the basal cells of the outer root sheath. Likewise the basal, proliferative populations of the nail matrix and nail bed do not express PAI-2; however, in many cases, the more differentiated progeny of these precursor populations do express PAI-2. These observations are consistent with our previous finding that PAI-2 is preferentially expressed by the suprabasal layers of human epidermis (Lyons-Giordano *et al*, 1994), and suggest that, in many of the epithelial cell types that comprise the epidermal appendages, PAI-2 can be considered as a differentiation marker.

In the outer root sheath cells of the hair follicle, the specifics of differentiation vary with the hair growth cycle, as does the expression of PAI-2. In anagen, the outer root sheath appears more complex than in telogen, consisting of a basal layer, a variable number of intermediate layers depending on position in the follicle, and a single layer of innermost cells. This innermost layer is the only subpopulation of the outer root sheath that expresses PAI-2. The cells of the innermost layer exhibit distinct ultrastructural features and a distinct keratin pattern compared with the outer layers of the outer root sheath or with the inner root sheath (Orwin, 1971; Ito *et al*, 1986; Ito, 1988; Rothnagel and Roop, 1995). These cells can be further distinguished from the

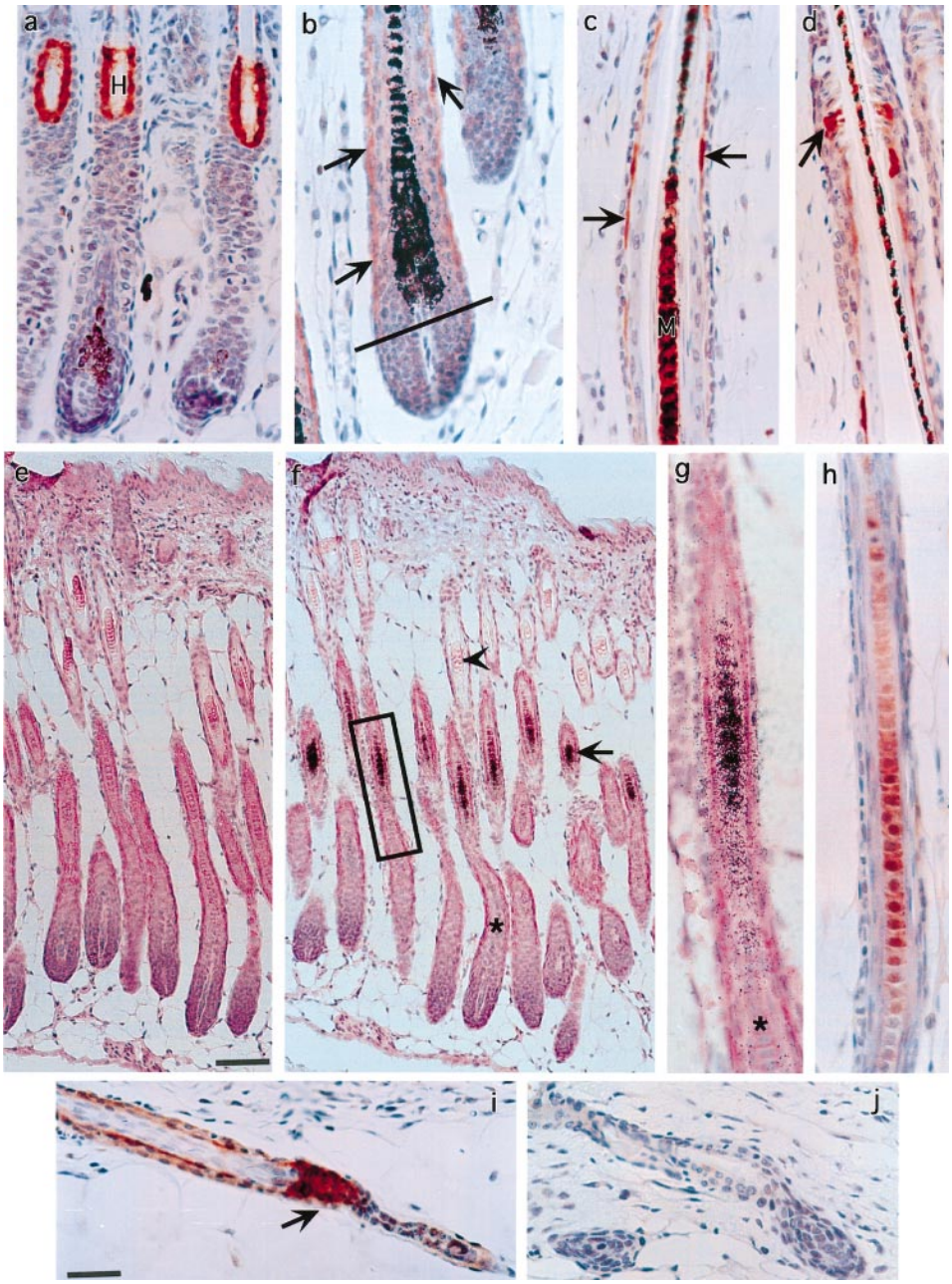

Figure 3. PAI-2 is expressed in anagen and catagen follicles. Immunostaining (*a–d*; *h–j*) or *in situ* hybridization (*e–g*) for PAI-2 was carried out, as in **Fig 1**, on skin taken from mice in the anagen (*a–h*) or catagen (*i*, *j*) phases of the hair growth cycle. (*a*) Follicles at an early stage of the second anagen, showing PAI-2 immunostaining in the outer root sheath of the club hair (H). (*b*) The lower portion of an anagen follicle, showing PAI-2 immunostaining (*arrows*) beginning around the critical line of Auber (marked with a black line) in the bulb, and extending upwards. Skin was taken from a black-haired mouse, hence the heavy concentration of melanin in the center of the follicle. (*c*) Longitudinal section of the mid-portion of an anagen follicle, showing PAI-2 immunostaining in a single line of flat cells (*arrows*), which is the innermost layer of the outer root sheath. Staining is also present in the medulla cells (M), alternating with the black pigment. (*d*) Section of the upper portion of an anagen follicle, where the inner root sheath is disintegrating and the innermost cells of the outer root sheath assume an irregular shape. Note PAI-2 immunostaining in the lower irregularly shaped cells (*arrow*), but not in the adjacent upper ones. (*e*) Sense control for *in situ* hybridization shown in (*f*). (*f*) PAI-2 *in situ* hybridization of skin from an 18 d old white-haired mouse, late in the first anagen phase. Strong signal for PAI-2 mRNA is found in a population of medulla keratinocytes in the mid-portion of the follicles (*arrow*). Signal is not detected in the less mature medulla cells in and just above the keratogenous zone (*), nor in the keratinizing medulla cells closer to the skin surface (*arrowhead*). The boxed area is shown at a higher magnification in (*g*). (*h*) PAI-2 immunostaining of an anagen follicle from a white-haired mouse, showing strong signal in the medulla cells in the mid-portion of the follicle, similar to the localization of PAI-2 mRNA. (*i*) PAI-2 immunostaining of a catagen follicle, showing strong staining in a group of cells (*arrow*) in the permanent portion of the follicle, beneath the retracting hair shaft. (*j*) A catagen follicle stained with normal rabbit IgG as a control for (*i*). *Scale bars*, (*a–d*, *g–j*) 40 μm; (*e*, *f*) 80 μm.

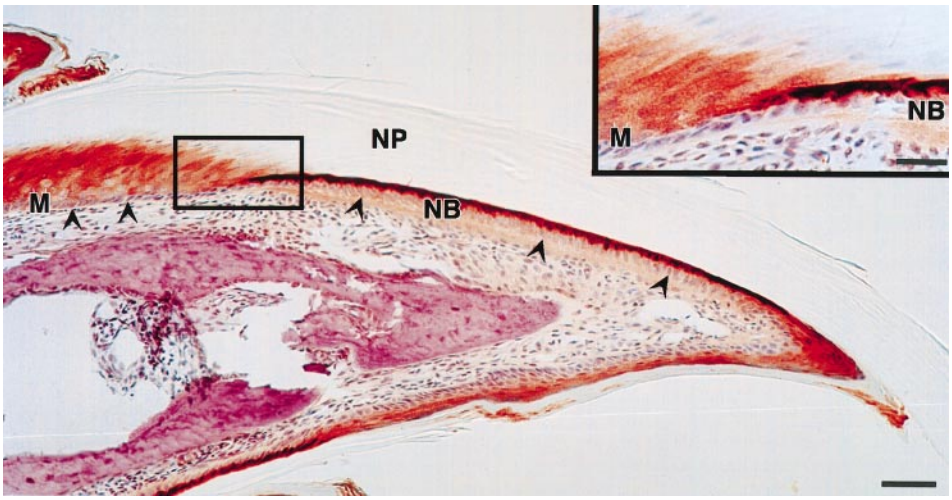

Figure 4. Differentiated cells of the nail apparatus express PAI-2. The nail region of the hind foot of a 3 wk old mouse was stained with anti-PAI-2 antibody. Note staining in the differentiating cells of the nail matrix (M), which gradually disappears as the cells lose their nuclei and keratinize to form the nail plate (NP). Note staining also in the uppermost, differentiated layer of the nail bed (NB). Staining is not detected in the basal cells (*arrowheads*) of either epithelium. *Scale bar*, 66 μm. The boxed area is shown at a higher magnification in the inset. *Scale bar*, 40 μm.

inner root sheath in that they lack trichohyalin (Rothnagel and Roop, 1995). Our findings are consistent with the idea that these cells are unique. At present there are two possibilities regarding the origin of the cells of the innermost layer of the outer root sheath: (i) they may be the terminally differentiated form of the outer root sheath cells, originating from the basal layer of epidermis; or (ii) they may represent a distinct lineage from the other cells of the outer root sheath and originate from the matrix keratinocytes of the follicular bulb (Orwin, 1971; Reynolds and Jahoda, 1993). Although we cannot distinguish between these two possibilities, our demonstration of a single line of PAI-2-positive cells emanating from the bulb and extending vertically nearly to the infundibulum favors the second possibility.

Regardless of the origin of the innermost cells of the outer root sheath, it is curious that this population is the only one in the inner or outer root sheath to express PAI-2. Based on a correlation between PAI-2 expression and the relative rates at which the inner and outer root sheath cells keratinize, we suggest that this may be consistent with a protective role for PAI-2 in prevention of premature keratinization and death. Specifically, Henle's layer, in the inner root sheath, cornifies early in anagen, prior to any other layer (Hashimoto and Shibazaki, 1976); the other layers of the inner root sheath quickly follow. Another characteristic of Henle's layer is that it is very tightly bound to the innermost layer of the outer root sheath (Reynolds and Jahoda, 1993), which contains PAI-2. Given these observations, we speculate that Henle's layer and the rest of the inner root sheath cells do not express PAI-2 because they keratinize quickly. In contrast, the innermost cells of the outer root sheath, which keratinize very late (Ito, 1988) and thus have a much longer postmitotic maturation time, require PAI-2 for protection from premature death.

In comparison with the outer root sheath of the anagen follicle, the telogen outer root sheath is less complex. In telogen, PAI-2 becomes strictly limited to a subset of relatively differentiated epithelial cells that surround the hair shaft, interdigitating with the fully cornified remnants of the inner root sheath. Although the PAI-2-positive cells in the telogen follicle are postmitotic, they remain viable and metabolically active, as evidenced by our ultrastructural and *in situ* hybridization analyses, and do not undergo terminal keratinization and death. As during anagen, expression of PAI-2 during telogen may confer protection to these cells from programmed cell death. It is likely that their continued viability is necessary to ensure that the hair shaft remains securely embedded in the follicle. In contrast to the human, murine hairs are essential for physical protection and thermo-regulation, and thus their retention is crucial to survival. PAI-2 appears to be constitutively synthesized by the more differentiated outer root sheath cells during telogen, as not only antigen but also mRNA for PAI-2 is detectable throughout the entire resting phase. These data imply a turnover and hence utilization of PAI-2.

Even in the brief degenerative stage between anagen and telogen, the follicle expresses PAI-2 in a manner consistent with our hypothesis of a protective role for this inhibitor against programmed cell death. The molecules responsible for initiation and regulation of programmed cell death during catagen have not been defined, although there is evidence that cytokines, including TNF-β and TGF-β, may play a role (Seiberg *et al*, 1995). In the environment of large-scale cell destruction that characterizes catagen, it is essential that the relatively small permanent population of cells in the follicle do not succumb to the death signals. We hypothesize that PAI-2 expression by a small group of cells in the catagen follicle protects them from the regulatory molecules that signal the demise of most of their neighboring cells. This hypothesis is consistent with the suggestion that TNF is a mediator of programmed cell death during catagen, as PAI-2 has been shown to protect cells lines from programmed cell death mediated by this cytokine (Kumar and Baglioni, 1991).

At present it is not possible to determine if the various follicular cell populations that we have identified as producing PAI-2 (i.e., the innermost cells of the outer root sheath during anagen, the epithelial cells at the base of permanent hair shaft during catagen, and the inner ring of epithelial cells during telogen) are distinctly derived populations, or alternatively are cycle-dependent manifestations of a common precursor. Appropriate markers do not exist to determine the fate of the innermost cells of the outer root sheath when anagen ends and catagen/telogen begins.

Although the hair and the nail serve different functions, many structural analogies can be drawn between these two tissues, with the nail apparatus similar in many ways to a hair follicle cut in half longitudinally. It has thus been suggested that the outer root sheath is analogous to the nail bed, with each anchoring a fully keratinized, hardened structure (i.e., the hair fiber and the nail plate, respectively) (Dawber and Baran, 1984; Baden and Kvedar, 1993). Interestingly, both the outer root sheath and the nail bed express PAI-2, specifically in the more differentiated cells of each, closest to the hair fiber and nail plate. A second structural analogy can be drawn between the hair fiber and the nail plate, which again corresponds to a common expression of PAI-2. The nail matrix keratinocytes, which form the nail plate, and the medulla cells, which form the central core of the hair shaft, both express PAI-2 during particular stages of differentiation. The nail plate is also analogous to the stratum corneum, as both terminal differentiation products show complete enucleation as well as autolysis of all cell organelles. Consistent with this analogy, PAI-2 is expressed in the granular layer of epidermis from human skin (Lyons-Giordano *et al*, 1994) and mouse footpad (Risse *et al*, unpublished observations). In all of these cases of terminal differentiation (i.e., nail matrix to nail plate, medulla cells to a component of the hair fiber, and basal keratinocytes to stratum corneum), numerous specific, complex differentiation products must be elaborated (Rogers and Harding, 1976; Jones and Steinert, 1996). Such terminal differentiation requires a long postmitotic maturation period, and too rapid a progression to cell death may not allow sufficient time to generate all the crucial products. We suggest that PAI-2 in each case may protect the postmitotic cells from entering prematurely into the final stages of terminal differentiation and death, thereby insuring a completely formed end product.

As discussed above, there appears to be a continuous utilization of PAI-2 by the differentiated cells of the outer root sheath of the hair follicle, as evidenced by the constitutive presence of both its mRNA and its protein throughout telogen. This raises the interesting question of what is the target proteinase for PAI-2? Classically, uPA has been considered as the physiologic target for PAI-2 (Andreasen *et al*, 1990, 1997), although there are several problems with this idea with respect to the epithelia of the hair and nail. First, we have not been able to detect uPA in the hair shaft or follicular epithelium, either by *in situ* hybridization or by immunostaining (Jensen and Lavker, 1996). uPA was not present during follicular neogenesis or during any of the phases of the naturally occurring or experimentally induced hair growth cycle. Secondly, uPA is secreted (Andreasen *et al*, 1997), and thus its inhibitor should also be secreted. PAI-2 is an unusual serine proteinase inhibitor in that, at least *in vitro*, it is largely accumulated within the cytoplasm of numerous cell types, including the keratinocyte (Jensen *et al*, 1995; Kruithof *et al*, 1995). Although we cannot determine from our present data the relative proportion of intracellular to secreted PAI-2, the staining patterns and intensity are consistent with a substantial amount of cytoplasmic PAI-2 in the follicular and nail epithelia. Finally, there is to date no evidence that plasminogen activator activity may mediate programmed cell death. On the contrary, Dickinson *et al* (1995) have provided evidence that uPA inhibition is not the mechanism by which transfected PAI-2 protects HeLa cells from programmed cell death. Hence, we believe that PAI-2 in the hair and nail may have a cytoprotective, cytoplasmic role, which involves inhibition of proteinase(s) other than plasminogen activators.

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